Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8768–8772, September 1995
Genetics

The urease locus of *Mycobacterium tuberculosis* and its utilization for the demonstration of allelic exchange in *Mycobacterium bovis* bacillus Calmette–Guérin

(allelic exchange)

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Communicated by Jonathan Beckwith, Harvard Medical School, Boston, MA, June 1, 1995

ABSTRACT **The *ureABC* genes of *Mycobacterium tuberculosis* were cloned. By using a set of degenerate primers corresponding to a conserved region of the urease enzyme (EC 3.5.1.5), a fragment of the expected size was amplified by PCR and was used to screen a *M. tuberculosis* cosmid library. Three open reading frames with extensive similarity to the urease genes from other organisms were found. The locus was mapped on the chromosome, using an ordered *M. tuberculosis* cosmid library. A suicide vector containing a *ureC* gene disrupted by a kanamycin marker (*aph*) was used to construct a urease-negative *Mycobacterium bovis* bacillus Calmette–Guérin mutant by allelic exchange involving replacement of the *ureC* gene with the *aph*::*ureC* construct. To our knowledge, allelic exchange has not been reported previously in the slow-growing mycobacteria. Homologous recombination will be an invaluable genetic tool for deciphering the mechanisms of tuberculosis pathogenesis, a disease that causes 3 × 10^6 deaths a year worldwide.**

Mycobacterium tuberculosis is an infectious agent responsible for more deaths worldwide than any other (1). The situation is worsened by the AIDS epidemic and the deteriorating socioeconomic conditions of an increased number of people (2). The recent application of molecular techniques to mycobacteria has led to the development of genetic tools such as transformation, transposition, and gene reporter technology that will be useful for investigating the physiopathology of the disease and the construction of new vaccines against tuberculosis (3–7). Although insertion of DNA by homologous recombination has been reported in fast-growing mycobacteria, allelic exchange has not been successful in slow-growing mycobacteria (8, 9). This has caused restrictions on the construction of defined mutants.

We investigated allelic exchange at the urease locus in mycobacteria because urease activity is easy to monitor and thus a potentially valuable marker. Urease (EC 3.5.1.5) is a nickel-containing enzyme that catalyzes the decomposition of urea to carbon dioxide and ammonia, the preferred nitrogen source for many bacteria (10). Urea is the principal nitrogenous waste product of mammals and is readily available in soil, water, and other environments. This probably explains the wide distribution of this enzymatic activity among Gram-positive and Gram-negative bacteria. Ureases have been implicated as virulence factors in various human and animal pathogens (reviewed in ref. 11). This is, for example, the case for *Proteus vulgaris*, which induces the production of stones in the urinary tract by raising the pH. Alkalinization of the urine by hydrolysis of urea to carbon dioxide and ammonia facilitates precipitation of struvite and carbonate–apatite (12). *Helicobacter pylori*, a bacterium that causes peptic ulcer, also hydrolyzes urea in this way. Ammonia production by urease enables *H. pylori* to colonize the stomach by neutralizing the acidic environment (13). Ammonia is also believed to contribute to the survival of *M. tuberculosis* inside the macrophage (14). By analogy with *H. pylori* and *P. vulgaris*, ammonia production by urease or transaminase may help *M. tuberculosis* to neutralize the acidic compartment of the phagolysosome.

The ureases of some Enterobacteriaceae, including *Klebsiella aerogenes* and *P. vulgaris*, consist of three different subunits: α, β, and γ, with molecular masses of approximately 70, 11, and 9 kDa, respectively. The composition of the complex is presumed to be $\alpha_2\beta_4\gamma_4$ (15). *Helicobacter* sp. urease differs from other bacterial ureases in that it is composed of only two subunits (16). The structure of jack bean (*Canavalia ensiformis*) urease is different from that of bacterial ureases: it consists of six identical 90.8-kDa subunits (17). Although the enzymes differ in the number of subunits, the amino acid composition of urease is highly conserved between plant and bacterial species. Moreover, in all species, urease activity requires the product of accessory genes involved in the synthesis and maturation of the nickel metallocenter (18, 19).

In this paper, we report the cloning, sequencing, and genomic mapping of the *ureABC* genes of *M. tuberculosis* and the allelic replacement of the *ureC* gene in *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), a mycobacterium belonging to the *M. tuberculosis* complex, but which can be safely handled.†

MATERIALS AND METHODS

DNA Manipulation. Restriction enzymes, the Klenow fragment of *Escherichia coli* DNA polymerase, and T4 DNA ligase were purchased from Boehringer Mannheim and used as recommended by the manufacturer. A 1-kb ladder of DNA molecular mass markers was from GIBCO/BRL. Chromosomal DNA was prepared as described (20).

Bacterial Strains. *E. coli* DH5α was grown routinely on solid or liquid Luria–Bertani (LB) medium (21). *M. tuberculosis* 103 (a clinical isolate) and *M. bovis* BCG Pasteur (1173 P_2) were grown in Middlebrook 7H9 (Difco) medium supplemented with Tween 0.1% or in Middlebrook 7H10 (Difco) solid medium. When necessary, antibiotics were used at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml.

PCR Conditions and Primers. Amplification by PCR was carried out according to the recommendations of the manufacturer. The 321-bp fragment used for Southern blotting

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Abbreviations: BCG, bacillus Calmette–Guérin; ORF, open reading frame.

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†The *ureABC* sequence reported in this paper has been deposited in the GenBank data base (accession no. L41141).

experiments was generated by using the GeneAmp (Perkin–Elmer) kit with P_1 and P_2 [P_1, 5′-AAG A(TC)(CG) CAC GAG GAC TGG GG(ACGT); P_2, 5′-GT(CT) (GC)A(GA) GT(GT) GTG GCA (CG)AC CAT-3′] as primers. PCRs consisted of 1 cycle of denaturation (94°C, 5 min) followed by 25 cycles of amplification consisting of denaturation (94°C, 1 min), annealing (49°C, 1 min), and primer extension (72°C, 1 min). The double crossover event was detected using the Expand Long Template PCR system (Boehringer Mannheim) and primers P_3 (5′-GGC GCA TCG GGT TTC AAA CTC CAC GAA GAC TGG GGA-3′) and P_4 (5′-GTG CGG GCG AAC CCC GAA AAA CAC CGG CTC CCA CAA-3′). The reaction consisted of 1 cycle of denaturation (94°C, 2 min) followed by 25 cycles of denaturation (94°C, 15 sec), annealing (65°C, 30 sec), and primer extension (68°C, 2 min).

DNA Hybridization. For Southern blotting experiments, 10 μg of genomic DNA was digested with 20 units of restriction enzyme and separated by electrophoresis through 1.0% agarose gels. Gels were processed and DNA was transferred to a nylon membrane (Hybond N+, Amersham) as described (22). The DNA was immobilized by UV crosslinking at 365 nm for 5 min. Membranes were prehybridized at 66°C for 2 hr in Rapid-hyb buffer (Amersham). The labeled probe (1 Ci/nmol; 1 Ci = 37 GBq) was denatured, added to the membrane, and incubated at 66°C for 2 hr. The membranes were washed three times at 66°C with 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS.

DNA Sequencing and Analysis. Sequences of double-stranded plasmid DNA were determined using the Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems) on a GeneAmp PCR system 9600 (Perkin–Elmer) and a run on a DNA analysis system, model 373 (Applied Biosystems). The sequence was assembled and processed using Gene Navigator (Applied Biosystems). The BLAST algorithm (23) was used to search protein data bases for similarity.

Construction and Screening of a Plasmid Genomic Library. Three micrograms of *M. tuberculosis* 103 genomic DNA was digested to completion with *Pst* I and separated on a 1.0% agarose gel. Restriction fragments of 2–6 kbp were excised from the gel, purified, and ligated to a dephosphorylated pBluescript KS− vector (24) linearized with *Pst* I. The ligation mixture was used to transform *E. coli* XL1-Blue, and 2000 ampicillin-resistant transformants were obtained. Plasmid DNA was prepared from 10 clones and all contained an insert. Colonies were transferred to nylon filters (Hybond N+, Amersham) and screened for hybridization under the conditions described above with an α-^{32}P-labeled 321-bp probe generated by PCR.

Construction of the *ureC* Disrupted Gene. One of the plasmids identified carried a 3.4-kbp *Pst* I insert containing the *ureC* gene. An *Eco*RI–*Sma* I fragment was isolated, its ends made blunt, and it was inserted into the *Hin*cII site of the pBluescript KS+ Δ(*Xba* I–*Cla* I) to give plasmid pJΔ64. The kanamycin-resistance gene (*aph*) of pUC4K (25) was isolated as a 1.3-kbp *Bam*HI fragment and inserted into the *Bam*HI sites in the *ureC* fragment in plasmid pJΔ64 to give plasmid pJΔ64K. Two micrograms of the resulting plasmid was linearized with *Sac* I and electroporated into *M. bovis* BCG as described (26).

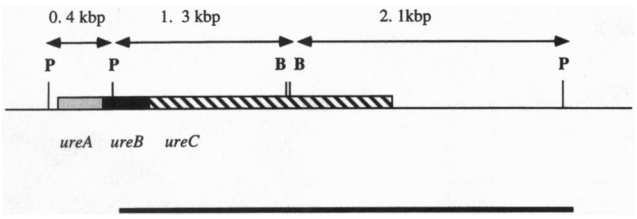

FIG. 1. Schematic organization of the urease locus of *M. tuberculosis*. Open reading frames are represented by blocks. Relevant restriction sites are indicated (P, *Pst* I; B, *Bam*HI). The *Pst* I probe used for Southern blot experiments is represented by a rectangle.

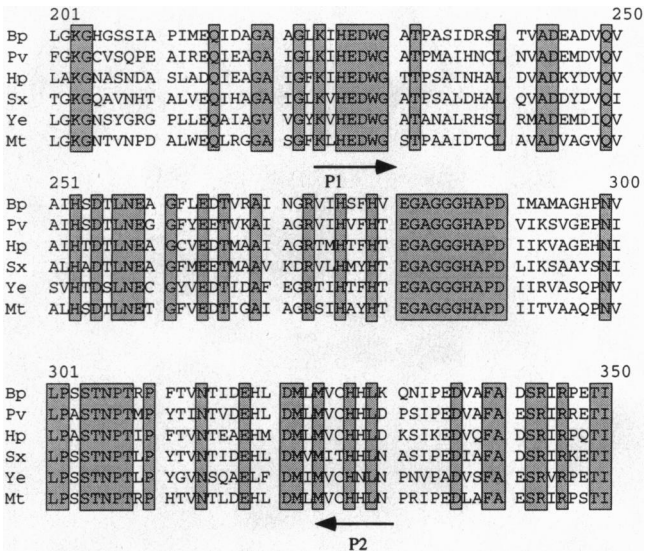

FIG. 2. Alignment of the amino acid sequence surrounding the active site of *ureC* of various bacterial species. This alignment was realized by using the PILEUP program of the University of Wisconsin Genetics Computer Group package. Identical amino acids are boxed. The probe used to screen the *M. tuberculosis* cosmid library was generated by PCR using P_1 and P_2 as primers. Bp, *B. pasteurii*; Pv, *P. vulgaris*; Hp, *H. pylori*; Sx, *Staphylococcus xylosus*; Ye, *Yersinia enterolitica*; Mt, *Mycobacterium tuberculosis*.

Detection of Urease Activity. Urease activity was demonstrated by detecting ammonia release from urea by following the pH with phenol red; 10^9 bacteria were suspended in 1 ml of urea/indol medium as described (27). A change of the color of the suspension to red was scored as urease-positive.

RESULTS

Cloning, Sequencing, and Genomic Mapping of *ureABC*. The region including the active site of urease is well conserved among different species (15). To clone the urease genes from *M. tuberculosis*, we therefore synthesized two degenerate oligonucleotides corresponding to the consensus protein sequences in this region. The two oligonucleotides were used as primers for PCR amplification with *M. tuberculosis* chromosomal DNA as a template. A PCR fragment of 321 bp, consistent with the fragment between the primer sequences in other species, was obtained; it was purified and used as a probe

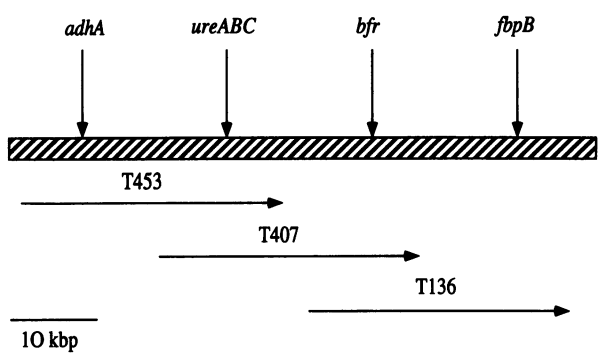

FIG. 3. Genomic mapping of the urease locus of *M. tuberculosis*. Genetic markers flanking *ureABC* are alcohol dehydrogenase A (*adhA*) and bacterioferritin (*bfr*). The size of cosmid clones was estimated from hybridization results.

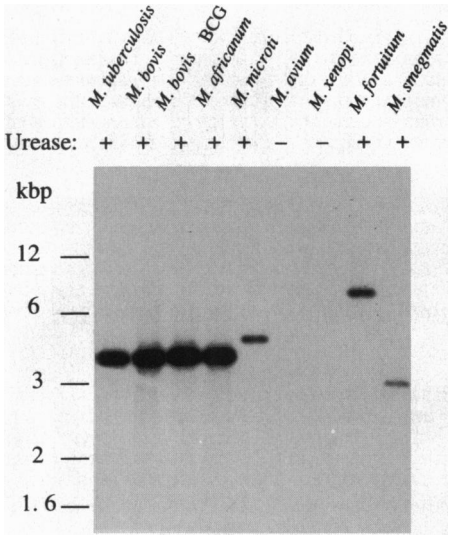

FIG. 4. Distribution of the *ureABC* locus among mycobacteria. *Mycobacterium* genomic DNAs were digested to completion with *Pst* I and analyzed by Southern blot under high stringency conditions with the probe depicted in Fig. 1. The urease phenotype is indicated above.

to screen a *M. tuberculosis* cosmid library (28). Two overlapping clones were found. The probe hybridized to an 8-kbp *Eco*RI fragment in these two cosmids. The 8-kbp *Eco*RI fragment was isolated and ligated into pBluescript SK+ and introduced into *E. coli* DH5α. The colonies were screened by hybridization with the 321-bp PCR fragment (*ure*) and a positive transformant was identified. Open reading frames (ORFs) encoding proteins similar to the two small subunits and the large subunit were located in this fragment by sequencing. They were designated *ureA, ureB*, and *ureC*, consistent with the nomenclature of enterobacterial urease structural genes (Fig. 1). The predicted molecular masses of the *M. tuberculosis* UreA, UreB, and UreC proteins are 11, 11, and 61 kDa, respectively. The UreA, UreB, and UreC proteins of *M. tuberculosis* and *Bacillus pasteurii*, another Gram-positive bacterium, showed 64%, 47%, and 57% amino acid identity, respectively. The similarities with *Rhizobium meliloti*, a Gram-negative bacterium, are 66%, 55%, and 62%, respectively. Fig. 2 shows the conserved region surrounding the active site of UreC between unrelated species.

The *M. tuberculosis ureABC* locus was mapped with respect to other genomic markers, by screening an ordered *M. tuberculosis* library for hybridization with the labeled 321-bp PCR fragment (*ure*). Two overlapping cosmids hybridized with the *ure* probe (Fig. 3). The *M. tuberculosis ureABC* locus was thus mapped to roughly 15 kbp from the *adhA* gene encoding the alcohol dehydrogenase A and about 15 kbp from the *bfr* gene encoding bacterioferritin (W. Philipp, personal communication).

Distribution of the Urease Genes Among Mycobacterial Species. We investigated the distribution of the urease locus among various species of mycobacteria by Southern hybridization. Genomic DNA of eight mycobacterial species (urease-negative and -positive species), including slow and rapid growers, was digested with *Pst* I and analyzed by Southern blotting (Fig. 4). Various sizes of restriction fragments hybridizing with the probe were found in the different species. All urease-positive strains gave a signal with the probe corresponding to the *ureBC* subunit. *Mycobacterium avium* and *Mycobacterium xenopi*, which are both urease-negative, did not hybridize with the probe. Surprisingly, the hybridization signal was weaker for *Mycobacterium microti*, a urease-positive species that belong to the *M. tuberculosis* complex, than for the other species belonging to this complex.

Inactivation of the Urease Activity of *M. bovis* BCG. To obtain a urease-defective mutant, we constructed a suicide vector containing a *ureC* gene disrupted by a kanamycin marker (the *aph* gene, Fig. 5). Two micrograms of this construct was linearized with *Sac* I and electroporated into *M. bovis* BCG. Kanamycin-resistant transformants were screened for the urease phenotype. Two of 50 tested (4%) scored urease-negative. One of the two was purified to single colonies and the phenotype was confirmed (Fig. 6).

Genotypic Characterization of the *M. bovis* BCG Urease-Negative Mutant. To characterize this *M. bovis* BCG urease-negative mutant, chromosomal DNA was prepared, digested with *Pst* I, and analyzed by Southern blotting using the *Pst* I probe (Fig. 7). The wild-type strain gave a single band at 3.4 kbp and the mutant gave two bands at 2.1 kbp and 1.3 kbp. This resulted from the *Pst* I sites of the *aph* gene integrated into the *ureC* gene (see Fig. 5). For further confirmation, PCR amplification was performed using primers flanking the *aph* gene (Fig. 8). The wild-type strain gave a band of 640 bp, whereas the mutant gave a fragment of 1940 bp resulting from the insertion of the 1300-bp *aph* gene into the *Bam*HI sites of *ureC*. Thus, the urease-negative BCG mutant was the result of allelic exchange involving replacement of the *ureC* gene with the *aph*::*ureC* construct and loss of the vector.

DISCUSSION

We report the identification and partial characterization of the *M. tuberculosis* urease genes, which have extensive similarity with the genes encoding other bacterial ureases. This urease locus consists of three genes—namely, *ureA, ureB*, and *ureC*. Interestingly, the amino acid conservation of these proteins between two Gram-positive bacteria is not higher than between *M. tuberculosis* and *R. meliloti*. The urease locus of *M. tuberculosis* has the same organization as most other bacterial loci: three ORFs encoding the three subunits (15). *ureABC* is probably an operon because the stop codon of the *ureA* gene overlaps the initiation codon of *ureB*, and the stop codon of *ureB* overlaps the initiation codon of *ureC*. In *Proteus mirabilis*

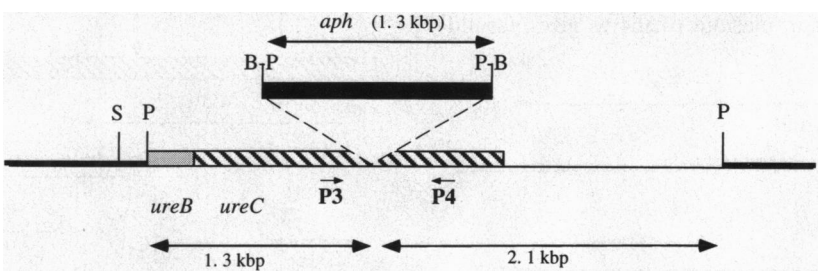

FIG. 5. Schematic organization of the *ureC* disrupted gene used for allelic exchange. Open reading frames are represented by blocks. Relevant restriction sites are indicated (P, *Pst* I; B, *Bam*HI; S, *Sac* I). The *aph* gene on a 1.3-kbp *Bam*HI fragment was inserted into the *Bam*HI site of *ureC* and is represented as a black rectangle. The solid bar represents the suicide vector pBluescript KS+. Primers used for amplification by PCR of the structure resulting from double crossover are depicted by arrows P_3 and P_4.

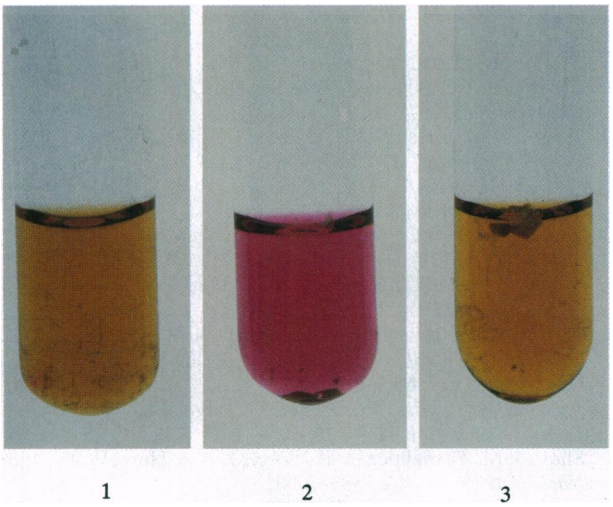

FIG. 6. Detection of urease activity. Tubes: 1, no bacteria; 2, *M. bovis* BCG; 3, *M. bovis* BCG urease-negative mutant.

and *Providencia stuartii* the three polypeptide subunits are transcribed as a single mRNA molecule (29, 30). No ORF with similarity to the accessory genes involved in the synthesis and maturation of the nickel metallocenter was found near *ureABC* in *M. tuberculosis*. It is interesting to note that *Mycobacterium smegmatis* and *Mycobacterium fortuitum*, which exhibit a urease-positive phenotype, gave weak hybridization signals with the *M. tuberculosis ureBC* probe. Surprisingly, *M. microti*, a urease-positive member of the *M. tuberculosis* complex, gave only a weakly hybridizing band of different size with the *ureBC* probe, indicating divergence from the others species of the *M. tuberculosis* complex.

We constructed a *M. bovis* BCG urease-negative strain by allelic exchange using a suicide vector containing a disrupted *ureC* gene. To our knowledge, allelic exchange has not been

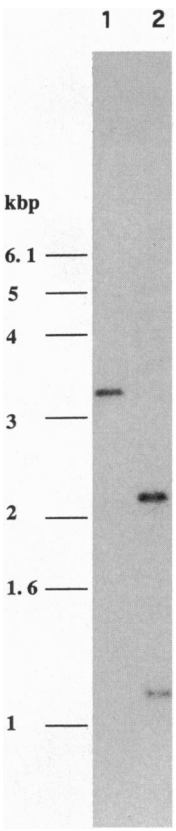

FIG. 7. Southern blot analysis of the *M. bovis* BCG wild-type and mutant strains. Lanes: 1, *M. bovis* BCG wild-type strain; 2, *M. bovis* BCG urease-negative mutant strain. Chromosomal DNA from the wild-type and the urease-negative strains was extracted, digested by *Pst* I, and analyzed by Southern blotting with the probe depicted in Fig. 1.

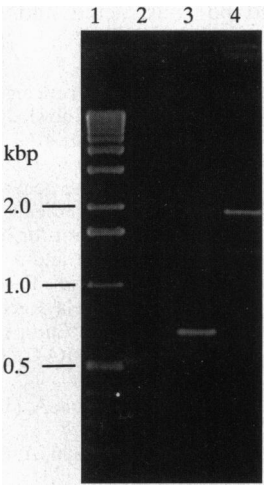

FIG. 8. PCR detection of the double crossover event. Lanes: 1, 1-kb DNA ladder molecular marker; 2, negative control; 3, *M. bovis* BCG wild-type strain; 4, double crossover mutant strain.

reported previously in slow-growing mycobacteria. Previous attempts to obtain allelic exchange in slow-growing mycobacteria used the *met* and the *uraA* genes but resulted in simple crossover or illegitimate recombination (7, 9). In contrast to these attempts, there are several probable reasons for successful allelic exchange at the *ure* locus. We choose a gene that is an excellent genetic marker and thus allows simple and rapid screening of the recombinants. Moreover, contrary to previous attempts (9), the linearized DNA molecules we used for transformation did not contain nonhomologous DNA at both ends. Another explanation for previous failure may be that the growth medium used did not support the growth of a *uraA* mutant of *M. bovis* BCG, leading to a counterselection of the double crossover event. It is also possible that the *met* and *uraA* loci are less favorable sites for homologous recombination than *ureC*. We also obtained urease-positive recombinants due to recombination by a single crossover (data not shown). PCR is a powerful tool for detecting gene replacement. In the double crossover mutant, the shorter (wild-type) fragment should be absent, whereas it will be present after a single crossing-over or illegitimate recombination event. Thus, PCR is particularly useful when the phenotype of the desired construct is not easily detectable.

We obtained gene replacement in a strain of the *M. tuberculosis* complex at about 4% (with respect to the kanamycin-resistant transformants). This is about 10 times less than has been reported for *M. smegmatis* (8). One of the key enzymes in recombination or repair in bacteria is the RecA protein. Recent studies have shown that the structures of the *recA* genes in *M. smegmatis* and in *M. bovis* BCG are dissimilar. The *recA* gene of pathogenic mycobacteria (including *M. bovis* BCG) contains a sequence coding for an intein (protein intron). The active RecA protein of pathogenic mycobacteria is obtained by post-transcriptional splicing (31, 32) and the presence of unspliced RecA blocks RecA activity (33). The low level of gene replacement in *M. bovis* BCG may thus be due to this unusual RecA maturation. The urease locus can be used to investigate homologous recombination in the *M. tuberculosis* complex. This system provides a powerful test to identify conditions improving the frequency of double crossovers. The system that we used involved selecting for transformation and recombination simultaneously. The development of thermosensitive vectors could improve efficiency of gene replacement by separating these two steps. Gene inactivation by homologous recombination would contribute to the study of pathogenicity of *M. tuberculosis* by allowing the analysis of mutants inactivated in candidate virulence genes. Moreover, auxotro-

phic mutants could be constructed and tested as vaccine candidates (34).

We thank J. Rauzier for automated sequencing of the *ureABC* locus and for help in sequence analysis. We acknowledge W. Philipp and S. Cole for the genomic mapping of the *ureABC* locus. We thank R. Ferrero and G. Guglielmi for their availability and very helpful discussions. We gratefully acknowledge the generous gift of mycobacterial genomic DNA by V. Pelicic, M. Picardeau, and D. Portnoï. We are grateful to A. Feuillet and A. Varnerot for help in manipulating slow-growing mycobacteria. A. Edelman and J. Batut are acknowledged for critical reading of the manuscript. F.-X.B. is a recipient of a grant from the Agence Nationale de la Recherche sur le SIDA (ANRS). This work was supported by a European Economic Community (EEC) Biotech program grant (BIO-CT92-0520).

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